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FINAL REPORT OF THE LAND-BASED TESTING OF THE BALPURE®-BALLAST WATER TREATMENT SYSTEM, FOR TYPE APPROVAL ACCORDING TO REGULATION-D2 AND THE RELEVANT IMO GUIDELINE

(APRIL – JULY 2009)

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Final report of the land-based testing of the BalPure®-Ballast Water Treatment System, for Type Approval according to Regulation-D2 and the relevant IMO Guideline (April – July 2009)

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1 Executive summary

The BALPURE® Ballast Water Treatment System (BWTS), produced by Severn Trent De Nora (STDN, LLC US), was tested according to the Regulation-D2 (D2-Standard), the IMO Guidelines for Type Approval testing (G8) and for approval of BWT systems that make use of active substances (G9) in the spring and early summer of 2009 in the harbour of the Netherlands Institute for Sea Research.

In general most of the requirements for testing were met and in several test runs environmental conditions were harsher than strictly required. The STDN Ballast Water Treatment System (BP-500) applies established chlorination technology to oxidize and disinfect water containing aquatic (invasive) organisms. Prior to adding the active substance (hypochlorite) the water was filtered through a 40 micron BallastSafe™ (BSFc) filter. In total 10 test runs were successful and the BWT system performed on average much better than stated in Regulation-D2 by achieving values for organisms well below the requirements of the D2 Standard. For many size classes of organisms the residual number of organisms was at least one order of magnitude lower than indicated numbers in the D2 Standard. This system should therefore be regarded as fast and an effective way of reducing the number of viable organisms in ballast water of ships, thereby minimizing the risk of new invasions originating from the ships ballast water.

The sediment load was reduced by the self-cleaning filter and remaining organisms were killed by the active substance chlorine. After chlorine treatment all organisms, including a substantial fraction of the bacteria population, disintegrated completely and the remaining debris was found as an amorphous structure or as dissolved organic carbon. To minimize potential regrowth of (micro) organisms a background level of chlorine was maintained (1 mg/L) until discharge. To reduce potential toxic effects of the chlorine at discharge sodium bisulfite was added prior to discharge.

No regrowth was observed for a period of 20 days in the presence of chlorine. Environmental acceptability tests however showed that the growth of organisms, mainly plankton, was not limited by the discharge water, after neutralization, indicating that the discharged water was still vital.

2 Zusammenfassung

Die BALPURE® Ballastwasser Behandlungsanlage, von Severn Trent De Nora (STDN, LLC US) wurde im Frühjahr und Sommer 2009 gemäß Regularien-D2 (D2-Standard), sowie der IMO Richtlinien zu Typzulassungstests (G8) und denen für eine Zulassung von Systemen, welche aktive Substanzen verwenden (G9) im Hafen des Königlich Niederländischen Meeresforschungsinstitut (NIOZ) getestet.

Generell wurden die allermeisten Anforderungen bezüglich der abiotischen Parameter des Testwassers und zur Organismendichte erfüllt. In den meisten Tests waren die Bedingungen schwieriger, als in den Richtlinien vorgesehen. Die BALPURE® Ballastwasser Behandlungsanlage (BP-500) verwendet bekannte Chlorierungsverfahren um Wasser zu oxidieren und dadurch potentiell invasiven Organismen abzutöten. Vor Zugabe der aktiven Substanz (Hypochlorit) wird das zu behandelnde Wasser über einen Feststofffilter (BSFc, 40 Mikrometerfilter) geleitet.

Es wurden 10 voneinander unabhängige Tests nacheinander, erfolgreich durchgeführt. Das System erfüllte alle Anforderungen der Regularien-D2 und der Richtlinie G8. Die Organismenanzahlen nach der Behandlung lagen deutlich unter den Anforderungen des D2-Standards, für einige Gruppen im Durchschnitt sogar um eine Größenordnung. Das System sollte demzufolge als effektive und sichere Möglichkeit zur Behandlung von Ballastwasser betrachtet werden, die dazu beitragen kann, weitere biologische Invasionen zu verhindern.

Die Sedimentfracht wurde durch die Filtration reduziert und die verbleibenden Organismen durch die aktive Substanz (Chlorverbindung) inaktiviert. Einschließlich eines substanziellen Anteils der Bakterienpopulation wurden fast alle Organismen vollständig zersetzt. Die Überreste der Organismen fanden sich lediglich in Form amorpher Strukturen und als gelöster organischer Kohlenstoff. Um Wiederaufwuchs von (Mikro-) Organismen zu verhindern wurde im Tank eine Konzentration der aktiven Substanz von ca. 1 mg/L bis zum Ablassen aufrecht erhalten. Um mögliche, negative Umwelteffekte hierdurch zu verhindern, wurde diese Restkonzentration beim Ablassen durch Zugabe von Natriumhydrogensulfit neutralisiert.

In Inkubationsexperimenten wurde in 20 Tagen kein Planktonwachstum im behandelten Wasser bei Anwesenheit von Chlor festgestellt. Gleichzeitig durchgeführte Umweltverträglichkeitstests (Zugabe von Zeigeorganismen und Planktonkulturen, Verdünnungsreihen) mit dem behandelten Wasser, nach der Neutralisierung, ergaben jedoch keine negativen Auswirkungen auf das Planktonwachstum. Diese Tests zeigten somit, dass das behandelte Wasser nach Neutralisation, d.h. so wie es in die Umwelt entlassen wird, das Wachstum gesunder Organismen nicht beeinflusst.

3 Summary table with results for the Type Approval Certificate of the BALPURE® BWT System(BP-500)

Land-based tests NIOZ	Reference & Treated			Reference			Treated		
salinity 23.9 PSU	Intake			Discharge			Discharge		
natural plankton	Average	min.	max.	Average	min.	max.	Average	min.	max.
total bacteria [counts/mL]	3.71+E6	1.78-+E6	5.65+E6	2.19+E6	0.41+E6	4.25+E6	4.03+E4	0.61+E4	7.09+E4
E. coli [cfu/mL]	-	< 0.1	0.14	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Enterococci [cfu/mL]	-	< 1	12	< 1	< 1	<1	< 1	<1	<1
plankton <10 µm [counts/mL]	6287	3283	11554	734	130	1485	1.5	0.7	4.4
plankton 10-50 µm [counts/mL]	1244	1046	1737	118	104	135	0.4	n.d.	1.5
plankton >50 µm [counts/m^3]	0.88+E6	0.39+E6	1.48+E6	3.54+E4	0.98+E4	7.22+E4	0.8	n.d.	4.3

Land-based tests NIOZ	Reference & Treated			Reference			Treated		
salinity 33.6 PSU	Intake			Discharge			Discharge		
natural plankton	Average	min.	max.	Average	min.	max.	Average	min.	max.
total bacteria [counts/mL]	4.38+E6	3.57+E6	5.32+E6	2.01+E6	1.48+E6	2.63+E6	1.79+E5	1.40+E5	2.73+E5
E. coli [cfu/mL]	-	< 0.1	1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Enterococci [cfu/mL]	-	< 1	100	< 1	< 1	<1	< 1	<1	<1
plankton <10 µm [counts/mL]	2790	2082	4070	814	305	2047	1.3	n.d.	3.7
plankton 10-50 µm [counts/mL]	1258	1002	1799	110	96	132	0.3	n.d.	1.48
plankton >50 µm [counts/m^3]	0.58+E6	0.15+E6	1.09+E6	1.25+E4	0.26+E4	1.91+E4	0.3	n.d.	1.0

Summary table of collected data covering the major groups of organisms at two series of test-runs for low and high salinity range, respectively.
n.d.: non detectable in sample.

4 Acknowledgements

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We also thank Scott Sorbet and Harold Childers for their technical assistance during the field tests and smooth operation of the BalPure®-BWT (BP-500) system.
We also thank Dr. Kai Trümpler, Mrs. Karin Sigel and Miss Mareike Wendland of the Bundesambt für Seeshifffahrt und Hydrographie, acting on behalf of the German Administration, for their excellent collaboration during this whole certification process

5 Introduction

Ships transport 5-10 billion tons of ballast water annually all over the globe (Endresen *et al.*, 2004). The ballast water is loaded with particulate sediment and an enormous variety of (living) organisms, which ranges from juvenile stages, larvae and eggs of fish and larger zooplankton (Williams *et al.*, 1988); (Carlton and Geller, 1993) to macroalgae, phytoplankton (Hamer *et al.*, 2000; Hallegraeff *et al.*, 1997), bacteria and viruses (Gollasch *et al.*, 1998). In general these organisms belong to the natural ecosystem in and around the port of origin but they might not be occurring naturally in the coastal waters and port of destination at the end of a ship's journey. In hundreds of cases around the world, this has resulted in severe damage to the receiving ecosystem and to human health, because these non-native organisms developed into a plague. This often has a high impact on the ecosystem and can cause economical damage (Hoagland *et al.*, 2002), as it results in a decrease of stocks of commercially valuable fish and shellfish species and occasionally outbreaks of diseases such as cholera (Ruiz *et al.*, 2000; Drake *et al.*, 2001). If action is not taken the problem of invasive species will increase in an exponential manner for several reasons. Ships are getting larger, faster and the amount of traffic across the oceans is expected to increase rapidly during the coming decades and therefore also the change of non-indigenous organisms to have large enough numbers for settling and expanding. Our effort to reduce pollution of ports and coastal waters also improves the quality of the aquatic environment in these areas and therefore increases the susceptibility to invasive organisms. Originally not intentionally meant but organisms in ballast water will experience favourable conditions for settling and growing. The problem of invasive species is considered as one of the 4 major threats of the world's oceans next to land-based marine pollution, overexploitation of living marine resources, and physical alteration/destruction of habitats

To minimize these risks for the future, the International Maritime Organization (IMO) of the United Nations has adopted the Ballast Water Convention in 2004 (Anonymous, 2005). The Convention states that finally ALL ships (>50,000 in number) should install proper ballast water treatment (BWT) equipment on board between 2009 and 2016. As a temporary and intermediate solution for the time being ships may reduce the risk of invasive species by performing ballast water exchange during their voyage when passing deep water (>200 m depth and 200 NM from the coast). Ballast water exchange faces many problems as to feasibility, safety and efficacy. For a large part of ships' voyages the required depth and/or distance to shore requirements are never met; BW exchange can affect the ships construction stability and in rough seas exchange is not possible *because of the risk to ship and crew. Treatment of ballast water is therefore considered* to be the best solution of reducing the risk of invasive species.

During the recent years numerous solutions for treatment of ballast water have been mentioned and tested with the ultimate goal to reduce the amount of organisms in ballast water (Rigby and Taylor, 2001). However, next to a high efficacy there is more needed for a BWT system to be good a system. Next to biologically effective the system should be practicable, environmentally acceptable and also cost effective.
Despite the fact that the treatment technology for drinking-, waste- and process water is well-developed none of these techniques is directly applicable to ballast water (Rigby *et al.*, 2001; MEPC 49/2/13, 2003). Besides reducing the load of organisms the sediment load should be reduced as well. There are also considerable differences in ships operation, types of ships, and the amount of space available for a ballast water treatment system on board and the way ships are operated. Ballast water treatment will develop into a new field of technology of its own with a commercial market estimated for the next 10 years in the order of 8 billion Euro (Haskoning, 2001).
As a primarily scientific research institute NIOZ is defining its role in the certification process of land-based trials of BWT systems as to study

1) the numerical abundance and biodiversity of organisms prior, during and after a treatment with the BalPure®-BWT system (efficacy of the BWT system),
2) to determine the viability status of the remaining organisms during discharge,
3) to assess possible environmental risks of discharging chlorine-treated ballast water by measuring residual effluent toxicity in order to determine latent effects, other than measured in specific toxicity tests conducted for the G9 (environmental impact).

This research strategy allows for more in depth testing, while it includes ALL organisms (from virus till whale) and not only the size classes as specified in the Convention D2-standard.

6 Description of the treatment facility

6.1 NIOZ Royal Netherlands Institute for Sea Research

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is part of the Netherlands Organization for Scientific Research (NWO). The institute employs around 200 people and the annual budget is approximately €20 million.
The mission of NIOZ is to gain and communicate scientific knowledge on seas and oceans for the understanding and sustainability of our planet. The institute also facilitates and supports marine research and education in the Netherlands and in Europe.

In order to fulfil its mission, the institute performs tasks in three specific fields.
Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. The institute also carries out research based on societal questions when this merges well with its fundamental work. The senior scientists at NIOZ all participate in international research projects.
Education: The institute educates PhD and other students of universities and schools for professional education. Together with universities NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists of NIOZ are also appointed as professors at the Dutch and foreign universities.
Facilitary services: *NIOZ invites marine scientists from Dutch and foreign institutes and* universities to write scientific proposals involving the institute's research vessels,

laboratories, and the large research equipment, which is often designed and built by the institute's own technical department.
The basic oceanographic **disciplines** studied at NIOZ are physics, chemistry, biology and geology. Multidisciplinary research is regarded as one of the main strengths of NIOZ.

More information on www.nioz.nl

Figure 1: aerial view of the NIOZ harbour (lower right), NIOZ laboratories (upper left) and TESO ferry (top).

6.2 North Sea Ballast Water Opportunity project

The Ballast Water Opportunity project (www.NorthSeaBallast.eu), originally an initiative of the BSH (Federal Maritime and Hydrographic Agency, Germany) and the Royal Netherlands Institute for Sea Research (NIOZ, Netherlands), involves all relevant stakeholders within the maritime sector in the North Sea region like governmental institutions, inter-governmental and non-governmental organisations, industry and scientific and technological institutes. This structure and participation offers a broad and sound base for the project in support of a succesful implementatin of the IMO Convention in the region, as North Sea, Skagerrak, and Kattegat form one ecological zone. Moreover, the project being one of the largest and most integrative in its kind, the objectives (investments) will become available as a model for other european maritime regions as well as across the globe. To facilitate this initiative funding was received from the North Sea InterreglVB (an ERDF program). For the embedding in a more global strategy the project is liaising with the Globallast II initiative of the IMO.

6.3 Portrait of Severn Trent De Nora, LLC., (producer of the BalPure® BWT-system)

More information on: www.severntrentdenora.com

Severn Trent De Nora, LLC., (STDN), is a joint venture subsidiary of Severn Trent Services and Gruppo De Nora. STDN is the world leader in the supply of on-site generation of biocide solutions to the power, marine, offshore industrial water disinfection markets. Over the past thirty years, STDN organizations have supplied 65% of the worldwide installed base of on-site generated hypochlorite. STDN draws upon the strength and global resources of Severn Trent Services and Gruppo De Nora. The joint venture was formed in 2001 by the combination of their respective subsidiaries, Exceltec International Corp. and DeNora Seaclor, S.r.l. The history of STDN includes: 1974- Diamond Shamrock Corporation, Electrolytic Systems Division formed. 1986- Acquisition of OMNIPURE by Diamond Shamrock Corporation. 1987- Formation of Eltech International Corp. (Sugar Land, TX). 1997- Eltech International Corp. name changed to Exceltec International Corp. 1998- Exceltec International Corp. acquired by Severn Trent Services. 2001- Severn Trent Services enters into joint venture with Gruppo De Nora. 2002- Severn Trent De Nora is formed. Established in 1923 by Oronzio De Nora, Gruppo De Nora is recognized worldwide as a leading supplier of electrochemical technologies for the production of chlorine, caustic soda and derivatives, as well as the largest worldwide supplier of noble metal-coated electrodes for the chlor-alkali industry and for the electrochemical industry in general. Energy saving and environmental protection are the Group's distinguishing technologies. Gruppo De Nora has built more than 500 plants for the production of chlorine and caustic soda and for the electrochlorination of industrial waters in 60 different countries.

Ballast water treatment - Technical Overview of BALPURE®

STDN has developed proprietary ballast water treatment systems that have been specifically designed to safely and economically eliminate the worldwide transfer of aquatic invasive species. BALPURE® technology is patented, proven, well understood and uniquely competitive based on thirty years of on-site hypochlorite generation equipment manufacturing and service to the off-shore and marine markets. The company has an experienced management team and has established key partnerships in naval architecture, marine engineering, systems engineering, fabrication, science, chemicals, distribution, and service.

In research underwritten by STDN at the University of Washington, the use of chlorination was proven effective in seawater on representative groups of all target organisms. Additional extensive shipboard testing is in progress conducted under a grant from the United States National Oceanographic and Atmospheric Administration (NOAA). Commercial land based testing has also been conducted on the BALPURE® system at the United States Naval Research Laboratory in Key West Florida as part of the Environmental Protection Agencies ETV program.

STDN as a United States based company is working to achieve global certification via two avenues. In the United States, the U.S. Coast Guard (USCG) Shipboard Technology Evaluation Program (STEP) is the first hurdle toward certification and STDN received acceptance in early 2009. European countries and much of the world is applying IMO proposed standards. STDN is pleased to work with the Royal Netherlands Institute for Sea Research (NIOZ) to conduct certification testing and the German Federal Maritime

Agency (BSH) who will act as the administrating agency during the certification and Type Approval process.

6.4 The test facility

The land-based tests were carried out at the Royal Netherlands Institute for Sea Research (NIOZ, www.nioz.nl), Landsdiep 4, 1797 SZ 't Horntje, Texel, the Netherlands, from March through July 2009.

The NIOZ test site is equipped with 3 coated concrete tanks of 300 m^3 volume each to simulate the ballast water tanks of the ship (Figure 2). The tanks were cleaned using pressure-washing after each run. Water samples were taken from bypasses of the standard piping used to fill and to empty the tanks or directly from the tank at outflow at ca. 30 c m from the bottom.

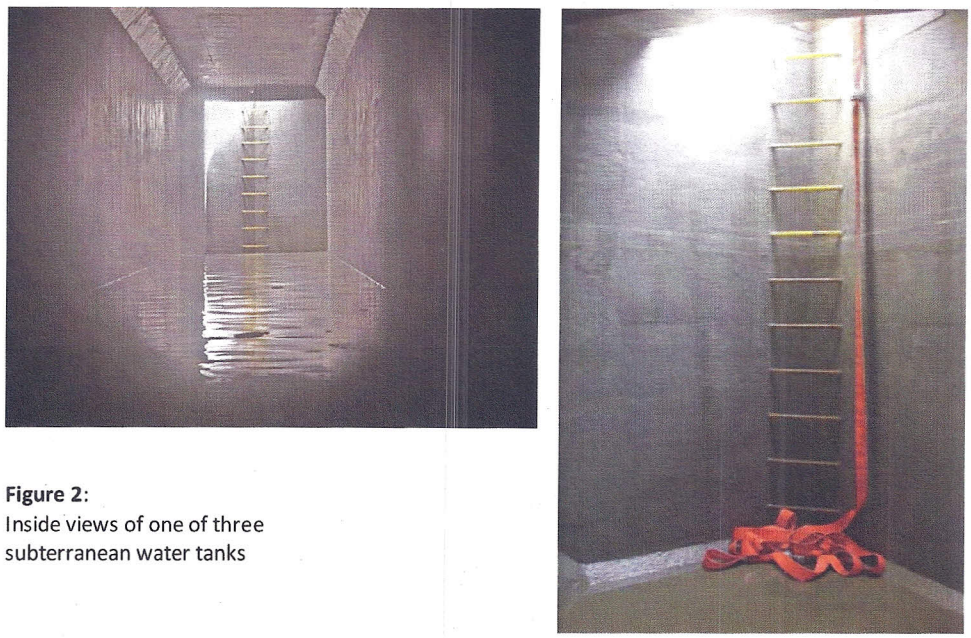

Figure 2:
Inside views of one of three subterranean water tanks

According to the requirements of the Guidelines G8, sampling points are fitted before the treatment system and directly after the system. Samples varying in volume from 500 mL up to 1 m^3 (clean IBCs) were taken using clean sampling containers. Sampling containers and all further handling of the samples were separated in a control and a treated set to avoid cross contamination by the active substance. The basic handling, such as concentrating, filtration and chemical analysis was done at the test site. Additional samples (1 to 10 L) were transported to the institute's laboratories for further special analysis. For re-growth experiments 10 L of sample was transported (Nalgene bottle) to a climate room for incubation experiments (ca. 12 – 15 °C; a light; dark regime of 16:8 h and 100 µmol quanta. $m^{-2}.s^{-1}$)

The BALPURE® BWTS was connected to a typical ballast water pump which was located in the NIOZ harbour. This is a pristine harbour with a direct access to the Wadden Sea and the origin of the test water changes with the tide. Furthermore, provisions were made to allow the addition of salt water and / or freshwater in order to adjust the salinity of the natural water of the NIOZ harbour to the required test conditions of brackish water

and marine water with a minimum of 10 PSU difference. A detailed description of the test installation is presented in figure 3

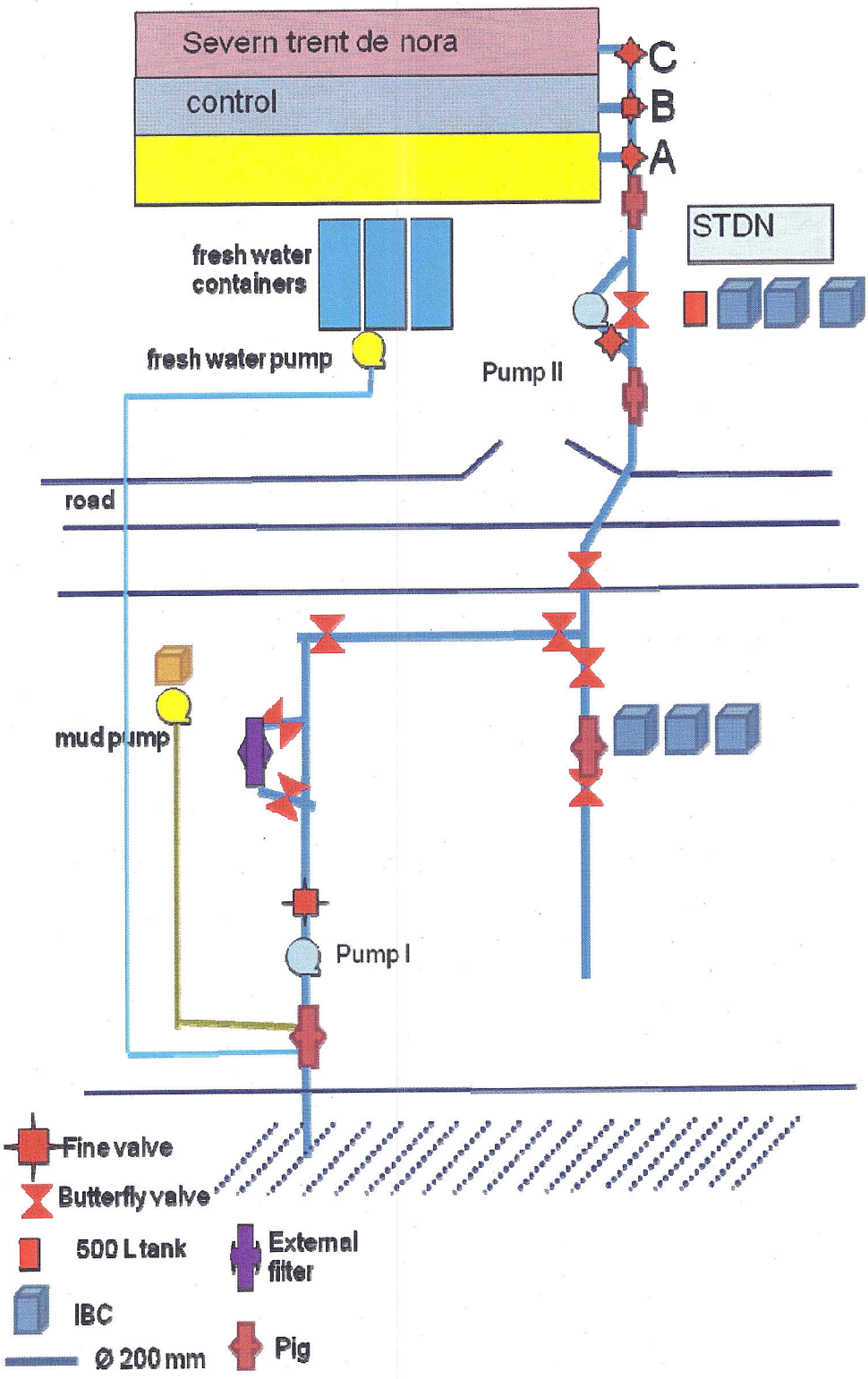

Figure 3: P&I diagram of the BALPURE® BWTS installation at NIOZ with points of sampling

6.5 Technical description of the BALPURE® BWTS (BP-500)

The STDN Ballast Water Treatment System (BWTS) applies established chlorination technology to oxidize and disinfect aquatic invasive species (AIS). Chlorination has a long history (80+ years) of being applied safely and economically for the control of microorganisms in industrial and municipal waters. The technology has found to be effective against a wide range of nuisance causing organisms. It is similar to a technology already approved by the IMO and type approved by the Republic of Korea.

The BALPURE® BP-500 BWTS provides a safe and simple electrolytic process for the on-site generation of dilute hypochlorite on demand without storage during the ballasting operation and the neutralization of residual oxidants during the de-ballasting operation. Sodium hypochlorite is generated by means of electrolysis of seawater. In this process, seawater flows through the electrolytic cells in a ratio of 1 part for every 100 parts of ballast water. The system can operate effectively in brackish water to 8 PSU. Because the amount of seawater required to effectively treat ballast water is small even fresh water ballast can be treated by BALPURE® BWT with supplemental salt or stored seawater in designated ballast tanks such as aft peak tanks. The current passing through the seawater causes the salt (NaCl) and water (H_2O) to form sodium hypochlorite (NaOCl) and hydrogen (H_2) as a secondary by-product. Hypochlorite solution and Hydrogen are produced and separated immediately upon exit from the electrolytic cells. The weak hypochlorite solution (1 g/L) is injected back into the ballast stream. Hydrogen that is separated from the hypochlorite is immediately diluted to less than 1% hydrogen by forced air blowers and discharged to a safe location. The hypochlorite is generated automatically on demand and is matched to the ballast flow rate and the oxidant demand of the ballast water. Initial oxidant concentration is dosed at 1.5 times the TOC concentration which remains effective for several days. To minimize potential regrowth of (micro) organisms a background level of chlorine is maintained (1 mg/L) until discharge. This will affect toxicity and by-product concentrations at compulsory sampling at day 1 and day 5 of the tests. To reduce potential toxic effects of the chlorine at discharge sodium sulfite or bisulfite is added prior to discharge. Sulfite when reacted forms sulfate which is present in seawater at concentrations 4,000 times stronger than the treated ballast water.

For this land based test, the water is filtered through a 40 micron BallastSafe™ BSFc Automatic Electric Filter, Model BSFc-H-1.6 prior to treatment. The unique sintered stainless steel screen technology enables it to have an unparalleled zooplankton removal rate. BallastSafe's filter features continuous cleaning of large volumes of dirt during ballasting without interruption, and a reversible screw system for smooth, reliable and rapid cleaning of the entire screen surface. The effectiveness of this filter has been successfully demonstrated in previous trials at NIOZ.

Figure 4: outline of BALPURE® BWT system

The BALPURE® Ballast Water Treatment System at NIOZ for land based testing is a full scale commercial available system of STDN and has not been downsized. The present configuration (BP-500) can be used for flow rates up to 500 m^3/h but was limited to a maximum of 200 m^3/h during the land-based tests. The BALPURE® BWTS is a self-monitoring system with an automated bypass, and has been reviewed for compliance with the class requirements of the ABS Americas Division classification society. All materials and specifications were selected in order to meet the class requirements of this classification society for ballast water systems and electric installations.
Subsequently, a second BALPURE® BWTS has been installed on a test ship (SeaRiver/Exxon-Mobil cargo tanker; American Progress).

The process for Basic Approval has recently started and a formal request to the MEPC has been made by the BSH.

The applied test protocols were communicated with the German Administration (Federal Maritime and Hydrographic Agency of Germany; BSH; Anonymous, 2009) and a short description of the various applied methods is included in the next section. During the certification process the whole practical procedure of intake and discharge has been witnessed at several occasions by national and international agencies.

7 Requirements to meet the Regulation-D2

According to D2-Standard of the IMO/MEPC Convention of 2004 (Anonymous, 2005) ships that meet the requirements of the Convention by meeting the ballast water performance standard must discharge:

1) Less than 10 viable organisms per cubic metre greater than or equal to 50 micrometers in minimum dimension;
2) Less than 10 viable organisms less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension and
3) Less than the following concentrations of indicator microbes, as a human health standard:
    1. Toxicogenic *Vibrio chlorerae* (serotypes O1 and O139) with less than 1 colony forming unit (cfu) per 100 millilitres or less than 1 cfu per 1 gramme (wet weight) of zooplankton samples:
    2. *Escherichia coli* less than 250 cfu 100 millilitres;
    3. intestinal Enterococci less than 100 cfu per 100 millilitres.

The present Standard-D2 is defined as a standard for the water characteristics at **discharge**. Furthermore, with exception of some indicator microbes (point 3) organisms < 10 µm are completely excluded. This is certainly an omission since this size class in particular includes numerous phytoplankton species characterized as Harmful Algal Blooms (HABs).
Nevertheless, the standard is clear with respect to the maximum number of organisms remaining present. On the other hand a proper definition of the dimensions of organisms is still subject of (academic) discussion. Moreover, as an operational definition for viable organisms the IMO is using: *organisms and any life stages thereof that are living*, but a more adequate (scientific) definition is: an organism that is able to complete its life-cycle, including reproduction (DNA replication). During the land-based tests cell viability in the context of both definitions was studied.

In addition to the (basic) requirements for the D2-Standard we have adopted a variety of methods and techniques to determine abundance, sizes and the viability status of different types of organisms. This includes also plankton < 10 µm other than bacteria (i.e. phytoplankton and viruses). Moreover, we extended our research effort to examine not only the fate of the organisms in the large-scale ballast water basins but also took subsamples for incubation under optimal growth conditions in our testing to study the growth potential of potentially remaining organisms or survival stages such as eggs, cysts or dormant cells over a longer period than the recommended 5 days. These later experiments allowed us also to study potential latent toxicity effects and therefore to determine the vitality of the treated water prior and after discharge.

7.1 Requirements to meet: guideline G8

Next to the D2-Standard two guidelines were developed by the IMO as a framework for approval of ballast water treatment systems (G8) and approval of the use of active substances in ballast water treatment systems (G9). For land-based testing MEPC 53/Annex 3 (Anonymous, 2005) and modifications as adapted at MEPC.174.58 (Anonymous 2008) were the basic framework of the present study. The most relevant parts will be presented below. These guidelines were generically designed to meet the conditions of a broad range of potentially effective treatment techniques to be tested in typical port and environmental conditions found across the globe. Most test protocols therefore require extensions of the test design to cover the specific aspects of the treatment. The land-based testing serves to determine the biological efficacy of the BWT systems under consideration for Type Approval under more or less controlled and replicable conditions. This is intended to ensure that the efficacy of the equipment is consistent and can be shown repeatedly. The test set-up should therefore be representative of the characteristics of the arrangements used and the type of environment the BWT system was designed for.

One of the main criteria in the G8 test requirements is the salinity range and related to this the differences in Total Suspended Solids (TSS), Particulate Organic Carbon (POC) and Dissolved Organic Carbon (DOC). This resulted in three main categories of test conditions (Table 1).

Table 1: Three different salinity ranges and minimum concentrations of TSS, POC and DOC in the water.

	Salinity			
Parameter	**> 32 PSU**	**3 – 32 PSU**	**< 3 PSU**	**unit**
Total Suspended Solids	> 1	> 50	> 50	mg/L
Particulate Organic Carbon	> 1	> 5	> 5	mg/L
Dissolved Organic Carbon	> 1	> 5	> 5	mg/L

Previous experiments and additional tests, as documented in the application for Basic Approval; (MEPC 58/2/2) showed that the BALPURE® BWT-System was not affected by differences in salinity. The physical step, filtration, will certainly not be affected by salinity but also the reactivity of the active compound (chlorine) will not be altered in the presence of high concentrations of salt. It was for this reason that the Type

Approval tests were conducted at the intermediate (3 – 32 PSU) and high salinity (>32 PSU) regions. Moreover, the only difference in composition of the test water between the freshwater and intermediate salinity water is the presence or absence of sea salt. All other minimum requirements for TSS, POC and DOC for these two water types were identical (Table 1).
The BALPURE® BWTS can treat ballast water of any quality listed in Table 1 and can use ballast water with salinity of ≥ 8 PSU to generate the necessary oxidants for ballast water treatment. A small supplemental salt or stored seawater source is required for ballast water with < 8 PSU for the slip stream to generate required oxidant amounts.
A further requirement is that the difference between the two salinity regimes should be at least 10 PSU. The test water, originating from the Wadden Sea, and the actual sampling did vary with the tide and as a result salinity was subject to variations. To assure the 10 PSU salinity differences it was decided to have the possibility of adding fresh surface water and upgrade coastal water of the North Sea by enhancing the salinity (brine solution of commercially available sea salt; ca. 18%). As target number the freshwater addition was adjusted to a salinity of ca. 23 PSU for the low salinity regime and ca. 33 for the high salinity regime. In practice ca. 15 % (v/v) of freshwater was added during the low salinity tests and about 4% of brine solution (Instant Ocean®), during the high salinity test runs. In order to compensate the dilution of the TSS by the freshwater some extra sediment (taking from a nearby mudflat) was added as well. These additions were made close at the pump site, to ensure proper mixing, with a constant flow rate and done during filling of the control and the treated ballast tank.

Biology

The guideline G8 also defines criteria for the number and diversity of the organisms to be met during Type Approval testing (Table 2). These criteria should be met for all three salinity regions.

Table 2: Minimal numbers and species diversity required at intake for different size classes and groups of organisms.

	Influent test water	
Parameter	**unit**	**Remarks**
organisms ≥ 50 micron	$> 10^5$/ m^3	at least 5 species from at least 3 different phyla/divisions
10 ≤ organism size ≤ 50 micron	$> 10^3$/ mL	at least 5 species from at least 3 different phyla/divisions
heterotrophic bacteria	$> 10^4$/ mL	not further defined

The test water should contain minimum densities of plankton which are typical densities encountered in the Wadden Sea during the annual spring bloom in April/May. With respect to the species diversity, the Wadden Sea is known for its natural richness in organisms and during the test period (April – July) indeed a large diversity in organisms, adults, juveniles, larvae, eggs, resting stages etc. was encountered.
An important aspect, so far not recognized in the guidelines (G8), when dealing only with natural populations of organisms in the influent of the test water is the natural seasonality of species and blooms. The actual onset of the spring bloom is characterized

by a dominance in phytoplankton, but usually lacks high zooplankton abundance. Only at a later stage zooplankton starts to increase in abundance, subsequently due to predation it will diminish the numerical abundance of (smaller size) phytoplankton component.
Furthermore, for the high salinity range, the composition of the organisms in the water resembles that of a typical oceanic environment. This implies an increase of smaller sized cells, down to the micrometer scale, and also a dramatic decline in the number of larger (>10 µm) organisms. So far this shift in community structure has not been accounted for when using natural plankton for testing.

Human pathogens

Table 3: Maximum allowed numbers of 3 groups of indicator microbes in the effluent test water on discharge. cfu: colony forming units

	Effluent test water	
Parameter	**unit**	**Remarks**
Toxicogenic *Vibrio chlorerae*	< 1 cfu/100 mL or < 1 cfu/ g wet weight of zooplankton	serotypes O1 and O139
Escherichia coli	<250 cfu/ 100 mL	
intestinal Enterococci	<100 cfu/ 100 mL	

Within the group of prokaryotic microbes only bacteria and more specifically the heterotrophic group (Table 2) has been defined by the standard but for completeness this should include all bacteria and presently also Archeae. While these microbes are part of the natural community in the aquatic environment the indicator microbes (Table 3), human pathogens are introduced as part of human activity and often associated with sewage discharge. In the present research all microbes have been included as a bulk parameter, the number of heterotrophs as a viable component as well as the viability of the whole microbial community has been determined.
Within the whole microbial community the number of heterotrophic bacteria was determined as well as *E. coli* and total enterococci. The test area of the institute is part of a tidal estuary of the Wadden Sea, which is essentially a pristine environment. Moreover, waste water treatment is highly developed in the Netherlands. Therefore, numbers of these human pathogens during the tests were to be expected to be low for most of the sampling period. On the other hand during the different treatment steps a significant amount of particulate organic material is transferred into dissolved organic carbon (DOC) which acts as an excellent substrate for stimulating growth of (heterotrophic) bacteria.

7.2 Experimental design

A variety of methods were applied to examine the biological efficacy of the BALPURE® BWT-system for the different categories of organisms during the two test series. For detailed description we refer to the outline of the official test protocols for the BALPURE® BWT-System (Anonymous, 2009). Sample handling and volumes were according to the description of the guideline for BWT testing (G8) or described in detail when these guidelines were insufficient or other considerations were taken into

account. Subsamples were taken randomly or throughout the whole filling procedure of the tanks. As indicated previously there was great emphasis on analysing the freshly collected samples and having multiple methods to examine numerical abundance and viability. Besides various biological samples there was also a basic set of physical and chemical parameters which were monitored prior, during and after discharge. A short description of each parameter and how it has been analysed is given below.

Physical and chemical properties of test water

Temperature

The water temperature was measured using a calibrated thermometer.

pH

The pH-level is measured using a calibrated pH-meter.

Salinity

For salinity ca. 250 - 500 mL water is sampled and stored at room temperature (glass bottles) until analysis by direct measurement in the laboratory at NIOZ. Salinity of the water was measured after each test cycle using a refractometer (Atago) calibrated against 0 and 33 PSU standard (sea)water. The accuracy of the salinity measurement is 0.5 PSU.

Dissolved Oxygen

The spectrophotometric method of the Winkler method (Winkler, 1888; Pai *et al.*, 1993); Reinthaler, 2006) was used to determine the oxygen concentration in the water. Samples were taken using gastight tubing which was specially fitted to the sampling tubing that was used to sample the ballast simulating tanks. The coded glass bottles are flushed at least three times their volume (ca. 120 mL) with water.
The sample bottles were stored in a dark container filled with water of the same temperature as the samples until further analysis at the laboratory. In the laboratory 1 mL H_2SO_4 is added prior to measuring the OD at 456 nm with a Hitachi U-3010 Spectrophotometer. The oxygen concentration was calculated using standards and expressed as µM O_2/L (or mg O_2/L = µM O_2 * 0.032). Since both salinity and temperature change over the season the oxygen concentrations were expressed as percentage relative to the natural saturation value for the given temperature and salinity.

DOC

The concentration of dissolved organic carbon (DOC) was measured according to Reintaler & Herndl (Reinthaler and Herndl, 2005). Samples for DOC (15 mL) were filtered through GF/C filters and sealed in pre-combusted glass ampoules after adding 50 µl of phosphoric acid (H_3PO_4). Sealed ampoules are stored at 4 °C. The DOC concentration was determined in the laboratory by the high temperature combustion method using a Shimadzu TOC-Vcpn analyzer. Standards were prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is < 3 %.

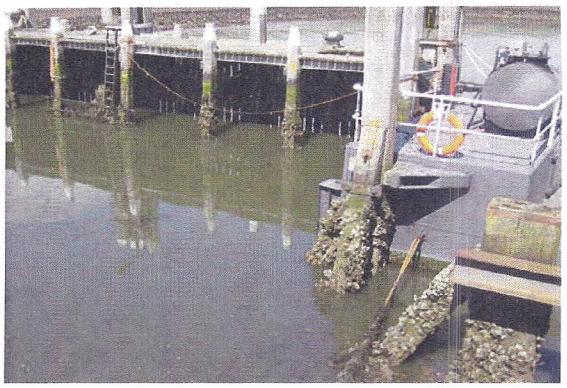

Figure 5: the challenge water with a high particle and sediment load.

TSS / POC (total suspended solids and particulate organic Carbon)

For TSS/POC pre-weighted glass fibre filters (GF/C) are used. Each filter was coded and stored in a clean Petri dish. The filtered volume was dependent on the particle load and concentration and type of organisms present in the water. The higher the total particle load in the sample, the smaller the volume that could be filtered before the filter clogs. Practical volumes were between 100 and 1000 mL per sample.
After filtration the filter was rinsed with fresh water (MiliQ) to remove sea salt. Filters were dried overnight at 60 °C and allowed to cool in a vacuum exicator before weighing. The total amount of suspended solids was calculated from the weight increase of the filter and averaged for the three replicates (mg/L).
Next, the filter is combusted at 500°C (overnight) and allowed to cool in a vacuum exicator and weighted again. The POC was calculated from the weight decrease between this measurement and the TSS weight.

Analytical determination of chlorine and related compounds

During each test cycle, the concentration of the active compound (Total Chlorine) was measured as soon as possible after filling of the treated ballast water tank, also at regular intervals during the 5 day hold period, and during discharge.

Chemistries that have been tested and verified by the United States Environmental Protection Agency (USEPA) are used to analyze the treated ballast water produced by the BALPURE® BWT-System.

Total Residual Oxidant (TRO) was measured as total chlorine using the Hach 2100p hand held Pocket Colorimeter II. The device employs the DPD Method accepted for reporting water analyses by the USEPA and is equivalent to USEPA method 330.5 for waste water and Standard Method 4500-Cl G for drinking water.

Figure 6: Example of sample collection point at tank 3.

Biology

The majority, but not the entire large size fraction (> 50 µm) consists of zooplankton, while the majority of the small size (10 - 50 µm) fraction consists of phytoplankton. Organisms > 50 µm are retained as recommended in the G8 guidelines MEPC 54/Inf.3 (using a modified Hydrobios net)..
Samples for the 10 - 50 µm fraction were collected from the effluent of the Hydrobios net. These samples were then filtered over a 10 µm sieve and fixated.
A second set of samples for this size class was taken and not separated from the organisms < 10 µm in order to include the fate of the smaller sized (phyto)plankton community as well and to avoid further damage of the plankton. The results of these samples were compared to the ones from the double filtered samples to evaluate the loss of organisms caused by processing the samples.

Sample sizes

During the land-based tests containers from 1 to 1000 L were used for sampling and/or storage. Samples were taken continuously and evenly during the whole process of filling or emptying the ballast water tanks. These containers were thoroughly rinsed or heat-treated prior to use. Samples for the human pathogens were taken in sterile (bar-coded) bottles provided by the bacterial test laboratory.

Figure 7: 1000 L container (IBC) with a 50 µm Hydrobios sampling net

Organisms > 50 µm

The samples are pre-concentrated over a Hydrobios 50 µm net resulting in an end volume of approximately 100 to 200 mL. The samples were transferred to the lab directly after sampling and Neutral Red was added in a ratio that yields an end concentration of 1:50,000. Staining time is 2+ hours. Neutral Red stains living organisms (Crippen, 1974; Fleming and Coughlan, 1978) distinctively and quite rapidly (less than one hour, figure 6). Therefore the viability assessment remains unaffected by the possible death of organisms during the staining or during sample analysis.
It is assumed that dead but physically intact organisms will also be found. Consequently a detailed inspection of each intact individual is needed to assess viability. This includes the staining as well as the detection of internal (heart, gills) movement. Organisms which were not intact are assumed to be dead.
Neutral Red is a reliable staining method for all major groups of organisms but inconsistent staining was found for bivalves. For this latter group movement (including

internal such as heart and gills for juvenile mussels) has to be used obligatorily to determine viability. This is dependent on the expertise of the person analyzing the samples. Therefore the same person analyzed all samples.

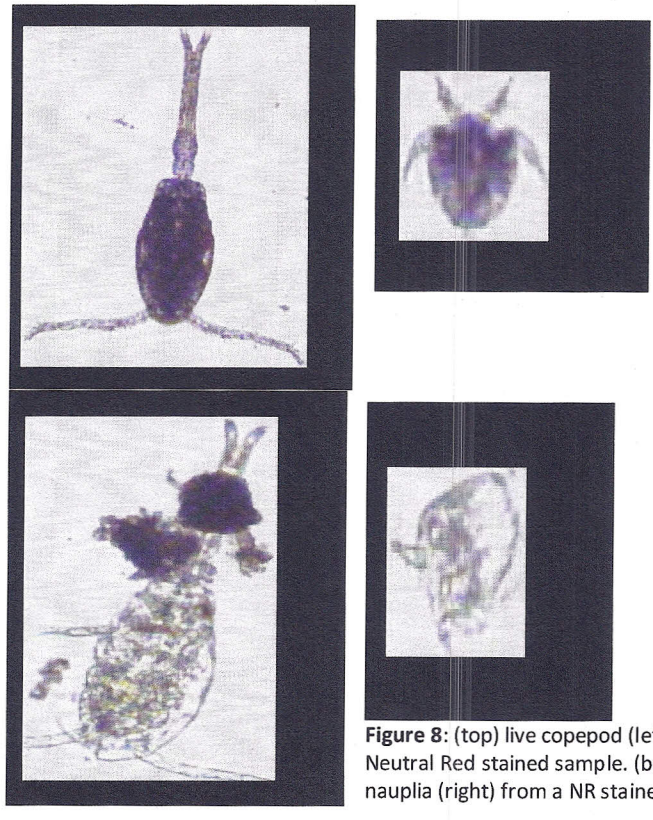

Figure 8: (top) live copepod (left) and nauplia (right) from a Neutral Red stained sample. (bottom) dead copepod (left) and nauplia (right) from a NR stained sample.

The samples are analyzed manually using a binocular with a 20x magnification for counting and up to 50x for species identification and measurements when necessary. For inter comparison a subset of samples was also analyzed using a semi-automated tool (FlowCam, Fluid Imaging Technologies; (Anonymous, 2001). Organisms need to be counted according to their size. Here organisms of 50 µm in minimum dimension are relevant. Several tests have shown that a single size bar is not efficient as viable organisms move in the counting chamber. Better results are achieved when the entire field of view is equipped with a size grid. The minimum dimension to measure will be adjusted to the specific organism groups.

Organisms < 50 µm

Samples for visual inspection of species number and diversity were pre-concentrated using a sieve made of a Hydrobios 10 µm mesh net using the 50 µm prefiltered sample (effective size range is 10 – 50 µm). The retained organisms were flushed into 50 mL Greiner tubes using filtered seawater and fixed with Lugols solution. Sample analysis was conducted by microscopic count with an inverted microscope at 200x magnification (method by Utermöhl). Since the Utermöhl is not suitable to asses viability counting was restricted to the structural integrity of organisms and therefore the presence of intact cells (Paerl, 1978). This method works for both zoo- and phytoplankton.
This size fraction was also covered by flow cytometry on basis of a single cell measurement (Veldhuis and Kraay, 2000) and PAM fluorometry, as a bulk parameter

(Schreiber *et al.*, 1993), using the intact and undisturbed samples. Besides numbers and sizes these two methods can be used to assess the cell viability (Veldhuis *et al.*, 2001; Veldhuis *et al.*, 2006) or in case of the PAM fluorometry also the photosynthetic efficiency of the phytoplankton.

Flow cytometry: For total organisms counts 3 mL of unfiltered sample water (reference and treated, each in triplicate) were analyzed using a calibrated flow cytometer. This yields the total number of particles (dead and live organisms as well as detritus) as well as their size range and the presence or absence of chlorophyll. For the counts exactly 1 mL was analyzed.
The size of the plankton was determined by comparison to standardized beads (10 and 50 µm). These beads were also used as standards to calibrate the performance of the flow cytometer.
For organism viability testing, on the level of the individual cell, SYTOX Green was added to 1 mL of sample water (control and treated, each in triplicate). After 15 minutes samples were analyzed using the flow cytometer for the presence of dead and/or live organisms (cf. Veldhuis et al, 2001, Casotti et al, 2005).

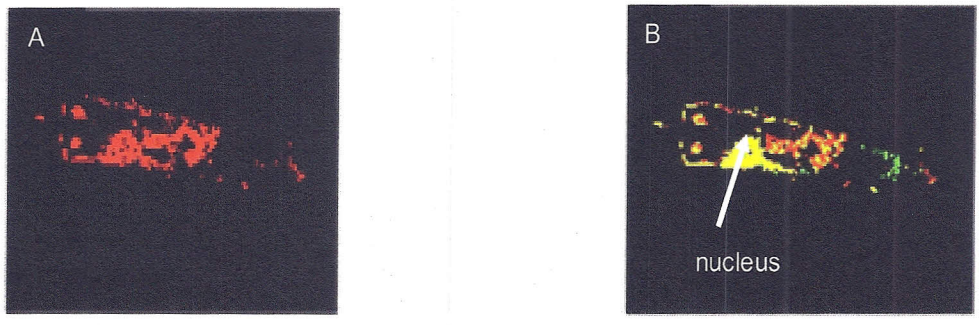

Figure 9: (A) Epifluorescence microscopic picture of a live phytoplankton cell. The red signal is due to the presence of chlorophyll and (B) a dead phytoplankton cell with a yellow/green fluorescence of the nucleus after staining with SYTOX Green.

PAM fluorometry: The photochemical efficiency of photosystem II of phytoplankton (providing an estimate of the general health of the algae) can be addressed using Pulse-Amplitude Modulated fluorometer (PAM-fluorometry) WALTZ- water PAM (Schreiber *et al.*, 1993). For this 3 mL of unfiltered sample water (control and treated, each in triplicate) are filled into a glass cuvette and analysed using the Pulse-Amplitude Modulated fluorometer. The instrument was calibrated against filtered seawater and a healthy fast-growing population of phytoplankton.

Bacteria

The classical method for counting bacteria in many applications is based on plating on selective media. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol and Del-Giorgio, 2000). As a result total bacteria were now determined by flow cytometry, using DNA-specific stains to get a more accurate bacteria number. In addition samples were taken at discharge for specific human pathogens and heterotrophic bacteria using a plate method.
A 1.5 mL water sample was taken and pipetted in a Cryovial (in triplicate) and formaldehyde was added as a preservative. Samples were frozen and stored at -50 °C until further analysis.

Upon analysis the sample is allowed to thaw completely. A subsample of 100 µl is taken, diluted with a TE-buffer, and the nucleic acid dye PicoGreen (MP) was added. Within 5 to 15 minutes after the addition of the stain the sample is analyzed using a flow cytometer (cf. (Gasol *et al.*, 2000; Veldhuis *et al.*, 1997). A known bacterial standard is used for calibration and counting.
The number of total heterotrophic bacteria was determined using a plate method as the number of colony forming units (cfu's) after incubation of the water at intake and discharge according to an international standard (NEN-EN-ISO 6222:1999 "Vitens laboratory bv" at Leeuwarden, RvA lab. no. L043)).

Human pathogens

The samples for microbiological analysis of the presence and number of human pathogens were taken in special bottles of 600 mL and send to "eurofins/C.mark bv" at Heerenveen (accreditation certificate: RvA lab. no. L043). All analyses were carried out according to NEN-EN-ISO standards.
These samples are sent to the laboratory immediately after sampling using a cooled transport container (4 °C). The analysis is carried out according NEN-EN-ISO 7899-2 for intestinal enterococci and NEN-EN-ISO 9308-1 for *E. coli* and related bacteria of the coli group as adopted for surface and waste water analysis in the Netherlands.